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Rhinoviruses Replicate Effectively at Lower Airway Temperatures

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Rhinoviruses are epidemiologically connected to the majority of acute asthma exacerbations; however, their ability to infect and replicate in the lower airways is disputed. A frequent argument against this possibility involves the temperature preference for rhinovirus replication, generally accepted to be 33°C, the temperature of the nasal passages. However, this argument is based on studies with a single rhinovirus serotype. In this study, differences in temperature preferences were evaluated between several serotypes and relative titers were determined than can be achieved at upper and lower airway temperatures. Rhinovirus serotypes 1b, 2, 7, 9, 14, 16, 41, and 70 were titrated in Ohio-HeLa cell cultures at either 33°C or 37°C. Possible selection by culture temperature was examined by continuous culture at 33°C and 37°C for 2–4 passages and subsequent titration at both temperatures. Finally, nasal aspirate samples derived from patients with wild-type rhinoviral common colds were cultured at 33°C and 37°C and RT-PCR was used to assess rhinovirus replication at each temperature. The majority of the serotypes and wild-type viruses replicated slightly better at 33°C than at 37°C. However, titers achieved after one or more replicative cycles at 37°C were still high enough to initiate infection. Furthermore, in some instances equal or even better replication was observed at 37°C. It is concluded that temperature preferences may vary between rhinoviruses and are not likely to be a prohibitive factor for infection of the lower airways. ***J. Med. Virol. 58:100–104, 1999.***

KEY WORDS: rhinovirus; respiratory virus; culture; common cold; asthma; temperature

INTRODUCTION

Initial isolation and cell culture of rhinoviruses (RVs) proved difficult in comparison to other respiratory viruses. It was achieved for the first time in 1960 using human embryo kidney cells, in rolled cultures, with low bicarbonate concentration, at 33°C [Tyrrell and Parsons, 1960]. Currently, RV culture is more successful using cell lines such as HeLa cells at 33°C. However, the temperature sensitivity of these viruses remains notorious. Only two studies in the 1970s have dealt with either sensitivity itself [Stott and Heath, 1970] or its underlying mechanisms [Killington et al., 1977] and only with a single RV serotype.

The ability of RVs to replicate at, or over, 37°C would not have been of significant interest, if convincing evidence showing that these viruses are important precipitants of asthma exacerbations had not come to light [Nicholson et al., 1993; Johnston et al., 1995]. A considerable amount of research effort has been directed toward the unraveling of virus-mediated asthma pathogenesis. Among several hypotheses, the possibility that RVs infect lower airway epithelial cells producing local immunologic and inflammatory responses is currently being scrutinized. Surprisingly, the low-temperature preference of RVs, in contrast to warmer lower airway temperatures, has frequently been used as an argument against this implication [Bardin et al., 1992; Busse and Gern, 1997; Corne and Holgate, 1997], without taking into account that published experiments have shown that the only examined serotype, rhinovirus-2, still propagated, although not optimally, at 37°C [Killington et al., 1977].

Therefore, in this study, the replication efficiency of several RV serotypes was compared at 33°C vs. 37°C. In order to assess whether continuous laboratory culture at 33°C is by itself a selective procedure, we also examined wild-type viruses direct from clinical samples and therefore not subject to possible in vitro temperature selection, and compared their temperature preferences with clinical scores of upper and lower respiratory symptoms that these strains had produced.

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MATERIALS AND METHODS

Virus Cultures and Titration

Human RVs 1b, 2, 7, 9, 14, 16, 41, and 70 were obtained from the MRC Common Cold Unit, Salisbury, U.K., the Public Health Laboratory Service, London, U.K., and the National Institute for Biological Standards, Herts, U.K. The identity of the viruses was confirmed by neutralization with specific antisera (ATCC, Rockville, MD). Ohio HeLa cells, obtained from the MRC Common Cold Unit, were seeded 2 days prior to infection at a density of 2×10^5/ml, reaching 60%–70% confluency at the time of infection. RVs were propagated in Eagle's MEM supplemented with 4% fetal calf serum, 2-mM L-glutamine, 10-mM HEPES (Gibco, Uxbridge, U.K.), 0.088% sodium bicarbonate, 30-mM magnesium chloride, 0.13% tryptose phosphate broth, and 40-μg/ml gentamycin (Sigma Chemical, Poole, U.K.). Cultures were performed in parallel, in two humidified, 5% CO_2 incubators at 33°C and 37°C. Titrations were performed in quadruplicate, by logarithmic dilutions of the viruses to $1{:}10^{-9}$. Titration plates were inspected daily for up to 7 days for the development of cytopathic effect (CPE), after which they were fixed and stained by 5% formaldehyde, 5% ethanol, 0.1% crystal violet in PBS, and the endpoint titer was read, defined as the highest dilution at which CPE was observed in half or more of the wells ($TCID_{50}$) and expressed as the inverse logarithm of this dilution.

Nasal aspirates of subjects with common colds and/or asthma exacerbations from a previous study [Johnston et al., 1995], in which RV infection had been confirmed by culture and reverse transcription polymerase chain reaction (RT-PCR), were retrieved from storage at −70°C. Clinical scores of upper and lower respiratory symptoms recorded by the subjects from whom these samples derived were also available [Johnston et al., 1995].

RT-PCR

RT-PCR of Ohio-HeLa cell lysates was performed as recently described [Johnston et al., 1993], with minor modifications. Briefly, to extract RNA 10 μl of sample were diluted 1:5 in ultrahigh-quality water (UHQ) followed by the addition of an equal volume of Trizol reagent (Gibco) and 1:10 volume of chloroform. RNA was precipitated with isopropyl alcohol, washed with 80% ethanol, vacuum-dried, and resuspended in UHQ. Reverse transcription was performed in a 50-mM Tris-HCl, 75-mM KCl, 3-mM $MgCl_2$ buffer, with 10-mM DTT, 0.4-mM dNTPs, 0.5-μg random hexamer primers (Promega, Southampton, U.K.), and 100 U of reverse transcriptase (Superscript, Gibco). The mix was incubated at 37°C for 60 min to yield single-strand cDNA.

PCR was performed in a total volume of 50 μl with 10 μl of cDNA, 10-mM Tris-HCl, 50-mM KCl, 0.1% Triton X-100 buffer with 1.5-mM Mg^{2+}, 0.2-mM dNTPs, 4.25-U Taq DNA polymerase (Promega), and 1.5 μM of primers OL27 (5′-CGGACACCCAAAGTAG-3′) and OL26 (5′-GCACTTCTGTTTCCCC-3′) (Oswel DNA Service, University of Southampton), which are complementary to the antisense RNA at positions 542 to 557 and 169 to 185 in the 5′ noncoding region of RV1b. The thermal cycle consisted of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 2 min, for 32 cycles, including a post-PCR extension step at 72°C for 4 min. A 380-base pair amplicon is generated, visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose gel. Parallel PCR amplification of beta-actin mRNA was used as a control of cDNA loading. Densities of PCR bands were measured by Band Leader V3.00 software in 300-dpi scanned images of ethidium bromide stained gels.

Experimental Design

RV temperature preferences were examined by four different sets of experiments.

Direct titration at 33°C or 37°C. To assess possible temperature-related differences in the minimum amount of virus necessary to initiate infection, RV serotypes were retrieved from the freezer and placed directly into two titration assays performed in parallel, at 33°C and 37°C.

Culture at 33°C or 37°C and subsequent titration at 33°C. We then proceeded to examine the ability of rhinovirus serotypes to expand in culture at either temperature. Viruses were cultured on Ohio-HeLa cells at both temperatures and harvested when CPE was well advanced by two freeze-thaw cycles. A constant volume (1 ml in a 25-cm^2 flask) of the lysate was used to reinfect freshly seeded HeLa cells for up to four passages, after which the expanded virus serotypes were titrated at 33°C.

Culture at 33°C or 37°C and titration at the same temperature. In order to exclude the possibility that viruses cultured at 37°C might be underestimated by the 33°C titration assay, a third set of experiments was performed: viruses were passaged twice at both temperatures, and the resulting viral preparations were titrated at the same temperature that they were cultured.

Short-term culture of wild-type viruses at 33°C or 37°C and RT-PCR on cell lysates. Since characterized rhinovirus serotypes have been exposed during their isolation procedure to temperature selection pressure in the laboratory, wild-type strains were also examined. Nasal aspirates, known to contain such strains, were put into culture at both temperatures for 72 hr, after which a semiquantitative RT-PCR was performed on the culture cell lysates. This approach was employed because wild-type rhinoviruses are usually present in very low titers in body fluids and increased sensitivity is advantageous in their early identification [Johnston et al., 1993]. The results of this experiment were correlated with clinical scores of upper and lower airway symptoms produced by these strains, in order to examine the possibility that differences in temperature

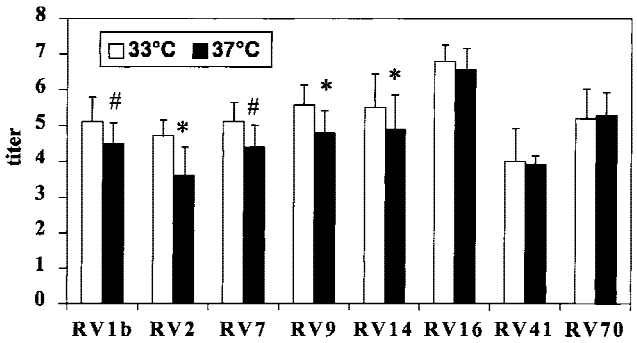

Fig. 1. Mean titers of human rhinovirus serotypes titrated directly from frozen at either 33°C or 37°C (n ≥ 4). Differences range from −0.1 to 1.1 log in favor of 33°C and are statistically significant in three of eight serotypes (marked *), marginal in two more (marked #), while three serotypes reached approximately the same titer.

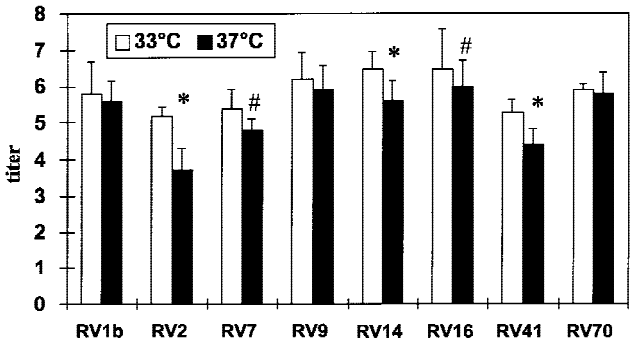

Fig. 2. Titers of human rhinovirus serotypes, continuously cultured at either 33°C or 37°C and subsequently titrated at 33°C (n ≥ 4). These are significantly in favor of 33°C cultured viruses in the case of RV2 ($P = 0.02$), RV14 ($P = 0.04$), and RV41 ($P = 0.04$); marginal for RV7 ($P = 0.1$) and RV16 ($P = 0.08$); while RV1b, RV9, and RV70 reached an almost identical titer at both culture temperatures. * denotes significant; #, marginal.

preferences may reflect different patterns of airway disease, i.e., rhinoviruses that might grow better at 37°C would produce increased lower airway symptomatology.

Statistical Analysis

Titration results are expressed as mean ± SD of 3–5 experiments. Mean titers were compared with a paired *t*-test. Simple regression analysis was performed to correlate clinical scores with PCR band densitometry results.

RESULTS

Direct Titration of Rhinoviruses at 33°C vs. 37°C

When rhinovirus serotypes were titrated directly from frozen at the two temperatures, small differences ranging from of 0.5 to 1.0 log in favor of 33°C were shown for most serotypes. These were statistically significant in the cases of RV2, 9, and 14, and marginal for RV1b and 7. RVs 16, 41, and 70 reached the same titer at both temperatures (Fig. 1).

Effect of Culture Under Different Temperatures

Similar to the above experiment, when viruses were passaged at 33°C and 37°C and subsequently titrated at 33°C, statistically significant differences in favor of 33°C were observed in RV2, RV14, and RV41, while RV7 and RV16 showed a marginal preference for 33°C. These differences were modest, as titers of viruses cultured at 37°C were only 10%–20% lower than those reached at 33°C. RV1b, RV9, and RV70 expanded to similar titers (Fig. 2).

Combination of Culture and Titration at the Same Temperature

To exclude the possibility that culture at 37°C could be selective of virus strains that grew better at that temperature and consequently be underestimated in a 33°C titration assay, viruses were also titrated at the same temperature as that at which they were cultured. It was confirmed that no such selection occurred during

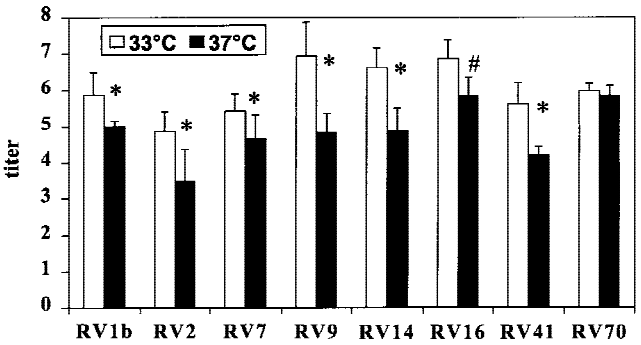

Fig. 3. Human rhinoviruses were cultured at either 33°C or 37°C and each virus preparation was subsequently titrated at the same temperature as it was cultured. Mean titers obtained from ≥3 experiments are shown. Significant ($P < 0.05$) differences in favor of 33°C are noted in RV1b, 2, 7, 9, 14, and 41 (*). RV16 shows a marginal (#) difference ($P = 0.06$), while RV70 titer is not affected by temperature.

the cultures. On the contrary, the growth advantage in favor of 33°C remained or increased, reaching up to 2 log (Fig. 3). Differences (33°C vs. 37°C) were statistically significant for RV1b (5.9 ± 0.5 vs. 5.0 ± 0.0, $P = 0.04$), RV2 (4.9 ± 0.6 vs. 3.5 ± 1.2, $P = 0.02$), RV7 (5.4 ± 0.4 vs. 4.7 ± 0.7, $P = 0.03$), RV9 (6.9 ± 1.1 vs. 4.8 ± 0.6, $P = 0.04$), RV14 (6.6 ± 0.5 vs. 4.9 ± 0.6, $P = 0.03$), and RV41 (5.6 ± 0.5 vs. 4.2 ± 0.2, $P = 0.02$); marginal for RV16 (6.9 ± 0.6 vs. 5.8 ± 0.3, $P = 0.06$); while RV70 showed no preference to either temperature (6.0 ± 0.0 vs. 5.9 ± 0.2).

Wild-Type Viruses

RT-PCR bands of wild-type rhinoviruses after short-term culture at either 33°C or 37°C were analyzed by densitometry. Uniform cDNA loading was confirmed by a beta-actin PCR performed in parallel. Peak densities of beta-actin showed less than 10% variability among all samples and rhinovirus peak densities were normalized according to the actin values. From eight wild-type rhinoviruses, three produced an increased signal at 33°C, four had nonsignificant differences,

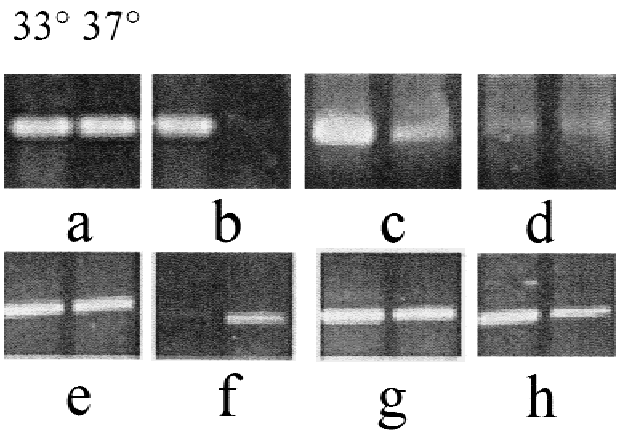

Fig. 4. Gel electrophoresis of RT-PCR products from HeLa cell lysates infected with wild-type rhinoviruses and cultured for 72 hr in parallel at 33°C and 37°C. Equal cDNA loading was verified by a simultaneous beta-actin PCR. When densitometry readings of PCR bands were compared, samples a, d, e, and g produced bands of equal density at both culture temperatures. A significantly stronger band was obtained for the 33°C cultured samples b, c, and h, while significantly more viral RNA is present in the 37°C culture of sample f ($P < 0.05$, n = 3).

while one had increased signal at 37°C (n = 3, $P < 0.05$ in all statistically significant cases) (Fig. 4). Clinical scores of upper and lower airway symptomatology, based on daily symptom cards recorded by the patients, were available from a previous study [Johnston et al., 1995]. To examine whether differences in temperature preference may be correlated with the variance in upper or lower airway symptomatology produced by these strains, simple regression analysis was performed. However, no statistical correlation between clinical scores and temperature-related differences in mean densities was noted (Table I).

DISCUSSION

Since their identification in the early 1960s, rhinoviruses have been shown to be the major cause of the common cold [Tyrrell and Parsons, 1960]. Although this disease is the most common ailment afflicting mankind, its benign nature has rather underestimated the role of rhinoviruses as pathogens, a notion reevaluated during recent years by the fact that these agents have been found to precipitate the majority of asthma exacerbations in children [Johnston et al., 1995] and adults [Nicholson et al., 1993].

Through this new perspective, a number of groups have considered the possibility that rhinoviruses may be able to replicate in the lungs, thus locally exerting their pathogenicity [Fraenkel et al., 1995]. Although evidence favoring this hypothesis is increasing, it is frequently argued that temperature preferences of rhinovirus replication could be prohibitive for such an infection to occur [Bardin et al., 1992; Busse and Gern, 1997; Corne and Holgate, 1997].

The ability of rhinoviruses to grow at 33°C has been one of the cornerstones for its initial isolation [Tyrrell and Parsons, 1960]. However, the assumption that optimal temperature is uniform for all serotypes and that it is imperative for virus propagation has been speculative, in contrast to data indicating that temperature sensitivity became considerable at 39°C, while at 37°C, differences were minor in the one serotype examined [Killington et al., 1977].

The results of this study are consistent with the notion that the temperature of the nasal cavities is optimal for growth of most rhinovirus serotypes. However, replication differences at 33°C and 37°C were always relatively small, for both the minimum amount of virus required to initiate infection and their expansion in continuous culture. In the cases of some serotypes, minimal or no differences were observed even after several passages. Interestingly, RV2, which was the only serotype used in the few temperature studies of the past, demonstrated the highest temperature sensitivity.

Virus yields at 37°C, irrespective of serotype, reached several orders of magnitude higher than those required for initiation of infection [Papadopoulos and Johnston, 1998]. Even though a progressive decline in titer at 37°C could theoretically result in eradication of the virus, this would not coincide with the time frame of its pathogenesis, i.e., a rapid increase in titer and subsequent elimination of the virus within days, probably through immunological mechanisms. More importantly, when we continued to propagate several serotypes at 37°C, no declining trend in titer was observed (data not shown). On the other hand, increased differences were observed in most cases, when culture and titration were both performed at either 33°C or 37°C. This probably indicates a cumulative effect of temperature on minimum infectious titer and expansion capacity. However, the fact that the titration assay, involving the host cells as well as the virus, is optimized at 33°C has to be taken into consideration. It is possible that increased metabolic activity of HeLa cells in culture at 37°C might affect their susceptibility to virus, in ways not reflecting in vivo events, such as small differences in pH or faster consumption of media nutrients.

In some cases, most notably RV70, no statistically significant difference between 33°C and 37°C was observed. It is therefore possible that optimal growth at 33°C is not a uniform characteristic of rhinoviruses. Nevertheless, it is more likely that in these cases growth differences were lower than the sensitivity of our assays. No serotype replicated preferentially at 37°C. We should, however, note that all serotypes have been isolated and expanded at 33°C for an unknown number of passages before reaching our hands, so they were potentially subject to considerable selection in vitro.

To examine further this possibility, clinical samples known to be rhinovirus positive from a previous study were cultured at 33°C and 37°C and assayed for viral mRNA with an RT-PCR. While most samples displayed either a stronger signal at 33°C or no significant differences, in one case the signal was considerably stron-

TABLE I. Clinical Scores of Upper and Lower Airway Symptomatology[a]

Subject	Upper airway score	Lower airway score	PCR band density difference (33°C–37°C, mean ± SD)
a	4	13	−12 ± 12.5
b	4	12	92 ± 11.1
c	4	5	47 ± 7.9
d	10	3	−6 ± 18.0
e	7	2	8 ± 10.1
f	7	0	−75 ± 42.1
g	12	8	14 ± 15.1
h	0	4	50 ± 19.3

[a]Compiled from daily symptom cards filled by patients with rhinovirus infection and obtained from Johnston et al. [1995]. These are compared to temperature-related differences in virus replication as assessed by short-term culture of nasal aspirates from these patients at 33°C and 37°C, followed by RT-PCR. Mean (n = 3) ± SD of differences in densitometry readings of PCR bands are reported. No correlation between disease pattern and temperature preference was observed.

ger at 37°C, indicating that rhinovirus strains preferring temperatures higher than 33°C may exist. Interestingly, this was the only virus with which no CPE at all was observed during the short-term culture. It is not impossible that current isolation procedures are not optimal for all rhinoviruses and some strains are underestimated. This is further supported by the fact that a considerable percentage of the rhinovirus-positive samples identified by PCR cannot be isolated in culture [Johnston et al., 1995]. Differences in optimal temperature for these strains did not correlate with upper and lower airway clinical scores of the patients from which they were isolated. This makes the possibility that lower airway disease may be associated with higher-temperature-preferring strains less likely; however, our sample was not big enough for conclusive results.

As in the case of most microorganisms, rhinovirus growth is the result of a number of factors, of which temperature is only one. While 33°C is the optimal growth temperature for most rhinoviruses, some strains are not significantly hampered at 37°C. Nevertheless, titers achieved at normal lower airway temperature are enough to support rhinovirus propagation. Furthermore, temperatures lower than 37°C can exist at lower airways as a result of rapid inspirations or cold environment [McFadden et al., 1982]. In conclusion, our data argue against the assumption that rhinoviruses cannot infect lower airways due to temperature sensitivity.

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